

PCT

WORLD INTELLECTUAL PROPERTY
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER

WO 9608231A1

(51) International Patent Classification: A61K 7/06, C07K 16/40		A1	(11) International Publication Number: WO 96/08231
			(43) International Publication Date: 21 March 1996 (21.03.96)
(21) International Application Number: PCT/GB95/02166		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).	
(22) International Filing Date: 13 September 1995 (13.09.95)		Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(30) Priority Data: 9418484.3 14 September 1994 (14.09.94) GB 9418547.7 15 September 1994 (15.09.94) GB			
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(54) Title: CONTROL OF HAIR GROWTH			
(57) Abstract A method for treating and preventing hair loss by topically administering an aromatase inhibitor to a mammal, including humans, on the area to be treated.			

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CONTROL OF HAIR GROWTH

Field of the Invention

5 The present invention relates to a method for determining the predisposition to hair loss and to preparations for the regulation of hair growth. These preparations are particularly applicable to the treatment of hair regression on the scalp and baldness and to depilatory agents in mammals, including humans.

Background to the Invention

10 Hirsuties and female androgenetic alopecia are common androgen-dependent problems. For example, each year about 200 women with these conditions are referred to the Department of Dermatology in Sheffield; patients are also referred to endocrinologists and gynaecologists. Hair is an important cosmetic asset and although these conditions are not physically disabling they can have a profound adverse affect on the quality of life.
15 Current treatments for hirsuties are, at best, only modestly effective. There is no worthwhile treatment for female androgenetic alopecia and, in most

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cases, management is limited to ensuring that there is no serious endocrine pathology and prescribing a wig for severely affected patients. Better treatments are needed but their development is hampered by our poor understanding of the basic biology. In particular, we do not understand why androgens stimulate hair growth over most of the skin but inhibit hair growth on the scalp.

Balding is a common alteration in the pattern of scalp hair growth. It affects both men and women although the degree of hair loss is less and tends to occur in a more diffuse pattern in women. Genetic factors are thought to be important and there are racial differences in the prevalence. There is strong evidence that androgen hormones are necessary for the development of balding but the mode of hormone action on the hair follicle is unknown. At present there is no satisfactory treatment. However, the following have some proven benefit;

15 Minoxidil

Topical application of minoxidil produces modest recovery in early balding in about 30% of subjects. Minoxidil is a potassium channel agonist in vascular smooth muscle. A number of other potassium channel agonists have been reported to stimulate hair growth (e.g. diazoxide, nicorandil) suggesting that this pharmacological property is relevant to their hypertrichotic effect. However the mechanism of action is unknown.

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Cyproterone Acetate (CPA)

This is a steroidal androgen receptor blocker which also has progestational activity. Several small trials indicate a small positive affect in female hair loss. CPA has to be taken orally and therefore cannot be used in men (whom it would feminise). There are no topical formulations available as far as we know.

Hair Surgery eg. hair transplantation, scalp reduction.

These techniques are not readily available and have very limited applications. Numerous other products have been promoted for the treatment of balding but, other than those listed above, there is no adequate clinical data on the results.

Development of new effective treatments for balding has been limited by our poor understanding of the basic biology, and the absence of suitable animal models for the routine testing of compounds.

Biological Background

Androgen hormones (e.g testosterone) are the major systemic influence on hair growth in man. In most body sites, such as the beard and pubic skin, androgens stimulate hair growth by prolonging the growth phase of the hair cycle (anagen) and increasing follicle size. Hair growth on the scalp does not require androgens but, paradoxically, androgens are necessary for balding on

↳ extragon
fct. hormone

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the scalp in genetically predisposed individuals (androgenetic alopecia) where there is a progressive decline in the duration of anagen and in hair follicle size. Men castrated before puberty fail to grow beards and do not go bald. If subsequently treated with testosterone about one third of male castrates will show balding [1]. Androgenetic alopecia is also common in women where it usually present as a diffuse hair loss rather than showing the patterning seen in men.

Current thinking is that testosterone is necessary for the development of balding but as only a proportion of men show balding, and there is no difference in circulating testosterone levels between bald and non-bald men, other factors must be needed for hair loss to occur. Until this work was carried out there was little indication as to what these other factors might be. Further evidence implicating androgens in balding (and growth of beard and body hair) is that genetic males who lack intracellular androgen receptors [testicular feminisation] fail to grow body hair and do not go bald in spite of high circulating levels of testosterone. It is generally believed that in most body sites, testosterone is metabolised in the skin to the potent androgen dihydrotestosterone (DHT) by the enzyme 5α - reductase before acting on hair growth. Genetic males with type 2 5α - reductase deficiency have normal or raised circulating testosterone levels but show a female pattern of body hair and sparse or absent beard growth. It is said that men with type 2 5α - reductase deficiency do not go bald but the evidence implicating this metabolic pathway in balding is less clear cut. Nothing is known of the

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prevalence of balding in the normal populations in which these cases have been reported and studies to date have failed to demonstrate the type 2 form of 5α - reductase in normal scalp. Administration of 5α - reductase inhibitors in macaques, a primate species which undergoes androgen-dependent scalp hair loss, has been reported to prevent and partially reverse the balding process but there is no clinical trial data published in humans. If DHT is involved in balding it would appear that the effect is derived from circulating DHT, not DHT synthesised in the scalp. This fails to explain why only a proportion of men show balding characteristics.

Stal T

10 Hair is a keratinised differentiation product of a highly proliferative population of epithelial cells situated at the base of the hair follicle (the hair bulb matrix). Hair is not produced continuously; individual hair follicles undergo a repetitive sequence of growth (anagen) and rest (telogen) known as the hair cycle. Control of the hair cycle is determined primarily within the

15 hair follicle itself but this intrinsic behaviour can be modulated by systemic influences mainly mediated by the endocrine system [2]. The hair follicle also contains a dermal component derived from embryonic mesenchyme. This comprises the dermal sheath surrounding the follicle and the dermal papilla which invaginates the base of the hair bulb. Transplantation studies in

20 rodents have shown the dermal papilla plays a vital role in hair growth. It is responsible for inducing growth and differentiation of follicular epithelium. It determines the follicle type (whisker, pelage etc.) and probably the diameter of the hair and there is indirect evidence that the dermal papilla is

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responsible for regulating the hair cycle [3,4]. A number of studies have suggested that, in follicles where androgens stimulate hair growth, the hormone acts primarily on the dermal papilla. Dermal papilla cells cultured from human hair follicles express saturable, high affinity androgen receptors and the receptor concentration is higher in cells from beard follicles than from occipital scalp follicles (a relatively non-androgen responsive site) [5]. Androgen receptors have also been demonstrated in the nuclei of dermal papilla cells in tissue sections by immunohistochemistry using a specific monoclonal antibody [6]. Significantly, there was no antibody staining of cells in the epithelial compartment of the hair bulb. Dermal papilla cells cultured from beard follicles also express type 2 5α - reductase whereas those from scalp follicles do not [7]. Type 1 5α - reductase is present in the scalp but men with 5α - reductase deficiency have normal levels of this enzyme. Therefore it seems likely that androgen action on the beard is mediated by DHT acting primarily on the dermal papilla but it is difficult to explain the inhibitory affect of androgens on scalp hair growth on the same basis.

In conclusion, the prior art is confusing in that it fails to explain the observed pattern of hair loss in a proportion of the population. It also provides no reasonable hypothesis on which to either predict a pre-disposition to hair loss and little basis upon which to base a systematic search for new and improved treatments.

Our investigations have instead lead us to study in some detail aromatase, a cytochrome p450 enzyme which catalyses the metabolism of androstenedione

DHT via 5 α reductase

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and testosterone to oestrone and oestradiol respectively. It is localised mainly in the ovary and the placenta and participates in the regulation of reproductive functions. Aromatase activity is also found in extra-gonadal tissues such brain, liver adipose tissue and genital skin fibroblasts. The aromatase pathway of androgen metabolism has received scant interest in hair biology and little is known about the effect of oestrogens on human hair growth. However a number of lines of evidence suggested to us that it may be important:

* In all mammalian species to have been studied (various rodents, dogs), oestrogens inhibit hair growth [8].

* Aromatase activity has been detected in plucked human scalp hair follicles [9].

* Aromatase protein has been detected in human scalp follicles by immunochemistry [10].

* We have recently found evidence of aromatase activity in vivo in rat hair follicles. This was an incidental finding in a study designed to measure the effect of a novel pre-oestrogenic steroid on sebaceous secretion. The compound in question is metabolised to oestrogen by the action of aromatase and will only act as an oestrogen in tissues which possess oestrogen receptors and aromatase activity. Topical application inhibited hair growth in the rat by about 35%.

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- * In genital skin fibroblasts, aromatase activity is stimulated by testosterone via an androgen receptor mediated mechanism [11].

As a result we formulated the following hypothesis:

5 In hair follicles in most body sites, testosterone acts as a pre-hormone and the response to testosterone is determined by which metabolic pathway predominates. In skin sites where 5α - reductase activity is high, such as in the beard, testosterone is metabolised to DHT which has a stimulatory action on hair growth. In scalp, testosterone is metabolised mainly via the aromatase pathway to oestrogens causing inhibition of hair growth. Aromatase activity is under receptor-mediated androgenic control; hence balding does not occur in testicular feminisation. The greater severity of balding in men compared with women can be explained by higher levels of oestrogen being attained in the male scalp by virtue of androgen stimulated aromatase activity. An analogous situation exists in the brain where 10 certain aspects of male behaviours in a number of avian and mammalian species are determined by oestrogens synthesised locally by androgen-dependent aromatisation of androgens [12]. This hypothesis is also able to encompass a role for 5α - reductase; as DHT is a more potent androgen than testosterone, circulating or locally 15 formed DHT can be a more effective inducer of aromatase.

20

Summary of the Invention

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In its broadest aspect, the invention concerns the realisation that aromatase, and more specifically androgenically controlled aromatase, has a role to play in hair loss.

5 According to a first aspect of the invention there is therefore provided the use of an aromatase inhibitor as a cosmetic agent.

According to a second aspect of the invention there is provided a method for treating or preventing hair loss comprising administering an aromatase inhibitor to an area to be treated.

10 In a preferred embodiment of this aspect of the invention the aromatase inhibitor comprises any one or more of the inhibitors listed herein. Alternatively the aromatase inhibitor may comprise an antibody raised against at least a part of said aromatase enzyme. The antibody may be polyclonal or monoclonal, monoclonal antibodies are preferred because of their homogeneity, however polyclonal antibodies are more easily produced
15 by injection of a suitable laboratory animal with at least a part of the enzyme and subsequently collecting serum therefrom and isolating specific sera using known techniques. Alternatively, monoclonal antibodies may also be produced using techniques well known to those skilled in the art.

20 According to a third aspect of the invention there is provided an antibody raised against the enzyme aromatase. Ideally the antibody is for use in preventing hair loss.

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According to the fourth aspect of the present invention, there is provided the use of an aromatase inhibitor in the manufacture of a preparation for the reduction in the regression of hair growth or in the alleviation of baldness. Such preparations induce, maintain or increase hair growth and can reverse, arrest or prevent the onset of baldness.

Preferably the preparation is formulated for topical application with suitable excipients in the form of a lotion.

Alternatively the preparation may take the form of a cream, shampoo, conditioner or spray.

Preferably the aromatase inhibitor is selected because it is readily absorbed from the preparation into the skin and into the appropriate skin cells. One means to achieve this is to use an aromatase inhibitor with lipophilic properties. One example of such an inhibitor is 4-hydroxy androstenedione. In principle any known or novel aromatase inhibitor can be used in such a formulation.

According to a fifth aspect of the invention there is provided the use of an agent adapted to selectively bind to an androgenic receptor, such that receptor mediated androgenic control of aromatase is suppressed or prevented, as a cosmetic agent.

In a preferred embodiment of this aspect of the invention said agent is an

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antagonist able to bind to said receptor but unable to produce a receptor mediated response.

5 Preferably the antagonistic agent may be synthetically produced or naturally occurring or alternatively a manipulated form of the naturally occurring androgen, for example, it may comprise a form of the naturally occurring androgen which is able to bind, and so block, the relevant receptor but unable to produce a receptor mediated response.

10 According to a further aspect of the invention there is provided a method for treating or preventing hair loss comprising administering an androgen receptor antagonist to an area to be treated.

According to a sixth aspect of the invention there is provided a medicament and/or cosmetic agent comprising an aromatase inhibitor and an androgen receptor antagonist.

15 In a preferred form of this aspect of the invention the aromatase inhibitor and the androgen receptor antagonist will be provided in a ratio that is related to the cellular concentrations of the corresponding aromatase and receptor.

Preferably said inhibitor is an anti-body, polyclonal or monoclonal, which has been raised against a part of the aromatase enzyme.

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According to a seventh aspect of the invention there is provided the use of an androgen receptor antagonist in the manufacture of a preparation for the reduction in the regression of hair growth or in the alleviation of baldness. Such preparations induce, maintain or increase hair growth and can reverse,
5 arrest or prevent the onset of baldness.

Preferably the preparation is formulated for topical application with suitable excipients in the form of a lotion.

Alternatively the preparation may take the form of a cream, shampoo, conditioner or spray.

10 Preferably the antagonist is selected because it is readily absorbed from the preparation into the skin and into the appropriate skin cells. One means to achieve is to use an antagonist with lipophilic properties. However, in principle, any known or novel androgen receptor antagonist can be used in such a formulation.

15 According to an eighth aspect of the invention there is provided a method for treating or preventing hair loss comprising administering, either successively or simultaneously, an aromatase inhibitor and an androgenic receptor antagonist.

In a preferred embodiment of the invention said aromatase inhibitor is any
20 one or more of the inhibitors listed herein such as, for example, 4-OH

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androstenedione.

Preferably the said androgenic receptor antagonist is for example, cyproterone acetate.

5 According to a ninth aspect of the present invention, there is provided a method of detecting if an individual is predisposed to hair growth regression or baldness by taking a sample of cells from various skin sites prone to baldness on the individual, culturing these cells, measuring their aromatase activity and comparing this activity with control values for people within a control group and values from people who have suffered from hair loss.

10 According to a tenth aspect of the present invention there is provided afore method for detecting whether an individual is likely to benefit from treatment with an aromatase inhibitor preparation. It is possible that only a proportion of those presenting at clinic will respond to such treatment because aromatase activity may not be the only mechanism responsible for
15 hair loss. This detection of abnormal levels of aromatase activity may indicate that this form of treatment is likely to be successful or worthwhile.

According to an eleventh aspect of the present invention there is provided the use of a pre-oestrogen in the manufacture of a preparation for the treatment of hirsutism. Such a preparation also finds an application as a
20 depilatory agent in what would otherwise be regarded as natural hair growth.

Description

If the above hypothesis is correct we would expect the following:

- * There will be a higher level of aromatase activity in skin sites prone to balding i.e frontal scalp and vertex than in non-balding sites such as the occipital scalp and the beard.
- * Skin aromatase activity will be induced by androgens via the androgen receptor.

In a preliminary study to investigate the above hypothesis we have measured aromatase activity in cultured human hair follicle dermal papilla cell lines and non-follicular skin fibroblasts from body sites with different responses to androgens. Aromatase was measured using the tritiated water released assay [13]. The results are summarised as follows:

- * Skin fibroblast lines from beard (N=5,) occipital scalp (N=5) and frontal scalp (N=7) all possessed aromatase activity. Enzymic kinetic values were established by incubating cells with the substrate (androstenedione) in a range of concentrations. The highest activities were found in cell lines from the frontal scalp i.e from a potentially balding site. Mean V_{max} (fmol/mg protein/hour) + / - SEM in beard, occipital and frontal cells were 892 + / - 155, 914 + / - 188 and 2,817 + / - 866 respectively. There was no difference in the K_m for

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androstenedione (mean = 25 nmol) between the three sites. This value is similar to that quoted for other tissues.

5 * Fibroblast aromatase was stimulated by pre-incubating cells with testosterone at physiological concentrations (10nmol). 19 cell lines were studied. Mean fold increase in aromatase activity (+ / - SEM) was 2.69 + / - 0.38 (p < 0.02 paired test). In separate experiments on three cell lines the response to a range of concentrations of testosterone and DHT was studied. Testosterone gave a twofold increase in aromatase maximal at 10nmol. At the same concentration in response to DHT was over twice as great (4.3 fold). This response to testosterone DHT was inhibited by cyproterone acetate (which blocks androgen receptors) indicating that androgen stimulation of skin aromatase is via an androgen receptor dependent mechanism.

15 * No aromatase was detected in hair follicle dermal papilla cells cultured through any of the above sites (fourteen cell lines). Testosterone failed to induce aromatase in dermal papilla cells.

 * Dexamethasone stimulated aromatase 10-20 fold in fibroblasts (six cell lines) and induced aromatase in dermal papilla cells (four cell lines).

20 * Fibroblast aromatase was inhibited by 4-OH androstenedione, a

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known inhibitor of aromatase in other tissues. Kinetic studies in three cell lines were consistent with irreversible competitive inhibition. 50% inhibition occurred at approximately 25nmol concentration.

Current thinking is that androgens are acting on the Dermal Papilla. The observation that there was no detectable aromatase activity in hair follicle dermal papilla cells would point away from our hypothesis. In contrast to the established line of thinking we believe that aromatase activity in non-follicular skin fibroblasts is more important and could account for the change in skin texture seen in balding sites.

The high levels of aromatase in fibroblasts from frontal scalp is consistent with our original hypothesis. The putative inhibitory effect of oestrogens on hair growth would need to derive from the surrounding dermis as aromatase activity was absent in dermal papilla cells. There is no evidence that androgens act directly on the hair follicle in the balding process and subjective observation of a balding scalp suggests that there is a general alteration in skin texture. It is possible therefore that inhibition of hair growth is a secondary response to more general changes in local biology of the skin. The response to testosterone and its inhibition by androgen receptor blockade is consistent with the absence of balding in testicular immunisation. DHT is a more potent inducer of aromatase than testosterone and this may explain the claimed absence of balding in 5 α - reductase deficiency. The absence of aromatase in dermal papilla cells when there are high levels in skin fibroblasts from the same tissue sample was a surprise finding and is the

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first evidence of a qualitative biochemical difference between these two cell types. The dissimilar response of dermal papilla cells and fibroblasts to testosterone and dexamethasone also suggests that aromatase is regulated differently in these two cell types. Tissue specific regulation of aromatase is well recognised [14, 15] and probably occurs at the level of gene transcription although non-genomic regulation has also been described in avian brain. These findings suggest that absence of aromatase activity is an important feature of dermal papilla function and that the maintenance of a "low oestrogen" environment may be necessary for hair growth to proceed.

It thus follows that treatment with an aromatase inhibitor will reduce or eliminate conversion of testosterone to oestrogen and consequently slow down or even reverse the balding process. The aromatase inhibitor can be formulated in a variety of ways. Although systemic administration is possible it is anticipated that side effects will be minimised by topical application. This can be achieved by way of a lotion or cream, including the usual excipients, creme base, stabilisers etc, or by way of a shampoo, conditioner, or spray. Such formulations are well-known to pharmacists skilled in the art and need no further detailing at this point.

Alternatively, by measuring the aromatase activity in cultured skin cells from a particular individual and comparing this activity with control values from people with a full head of hair and values from people who have suffered hair loss, it is possible to determine if an individual is predisposed to hair growth regression or baldness.

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It is also possible to use a combination of two or more aromatase inhibitors in the same preparation in order to maximise its activity. Such inhibitors may be specifically designed for the purpose or may be chosen from the non-comprehensive list of known inhibitors given below;

- 5 In summary the preparations described above induce, maintain or increase hair growth and reverse, arrest or prevent the onset of baldness. They may thus be particularly applicable to people pre-disposed to such conditions.

It will be appreciated that if the aromatase enzyme is important in the regulation of certain aspects of hair growth, it is also possible to increase
10 oestrogen concentrations and thus reduce hair growth, by treatment with a pre-oestrogen. Such compounds are known to those skilled in the art and can be formulated for topical application using suitable conventional means. They are converted *in vivo* to oestrogen which can then exert its known effects on the hair cycle.

- 15 Topical application of a pre-oestrogen is particularly usual for the treatment of hirsutism and as a depilatory agent. Again, assaying the skin cells for the presence of aromatase activity can give a prior indication that such treatment is likely to be effective.

20 Preferably the aromatase inhibitor or other active component is in substantially pure pharmaceutical form. Administration may be by any suitable route but the transdermal route is preferred. Topical preparations

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may be in the form of a cream, shampoo, conditioner or spray.

Liquid preparations may be in the form of, for example, aqueous or oily suspensions, solutions, emulsions, or may be presented as a dry product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may contain conventional additives such as suspending agents, emulsifying agents, non-aqueous vehicles and, if desired, conventional flavouring, perfuming, or colouring agents.

Administration is also possible by subcutaneous injection, fluid unit dose forms being prepared containing a compound of the present invention and a sterile vehicle.

The choice of pre-oestrogen(s) should be partly dictated by their ease of absorption through skin.

AROMATASE INHIBITORS (not a complete list)

Aminoglutethimide

4-OH androstenedione

6-hydroximino-androstenedione

9, 10-dimethyl 1,2-benzanthracene

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1,4,6-androstatrien 3,17-dione

Atamestane

Exemstane

Fadrozole

5 Vorozole

1,2,4-triazole-3-alanine

Rogletimide

10 In this context an inhibitor is any agent capable of preventing the aromatase enzyme from carrying out its normal function. This includes partial and full antagonists and also encompasses antibodies raised against at least a part of the aromatase enzyme. The antibodies may be polyclonal or monoclonal and can be produced using techniques well known to those skilled in the art. Manipulated forms of naturally occurring androgens may also be used.

Conclusions and Summary

15 Androgen hormones are necessary for the development of male balding but the mechanism of their action on scalp hair follicles has been obscure. We have proposed that balding is caused by oestrogens which are synthesized

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locally in the scalp from circulating androgens (testosterone, androstenedione) by the action of the enzyme, aromatase. Oestrogens are known to inhibit hair growth in other mammals. Also, aromatase activity is androgen dependent in some tissues e.g. brain. To investigate this hypothesis we have studied aromatase in skin from balding and non-balding male scalp, female scalp and male beard (where androgens stimulate hair growth). These studies have used cultured skin fibroblasts, cultured hair follicle dermal papilla cells and skin tissue.

Results

- Skin fibroblast lines from beard (n=5), occipital scalp (n=5) and frontal scalp (n=7) all possessed aromatase activity. Enzyme kinetic values were established by incubating cells with the substrate (androstenedione) in a range of concentrations. The highest activities were found in cell lines from the frontal scalp i.e from a potentially balding site. Mean V_{\max} (fmol/mg protein/hour) \pm SEM in beard, occipital and frontal cells were 892 ± 155 , 914 ± 188 and 2817 ± 866 respectively. There was no difference in the K_m for androstenedione (mean = 25nmol) between the three sites. This value is similar to that quoted for other tissues.

Fibroblast aromatase was stimulated by pre-incubating the cells with testosterone at a physiological concentration (10nmol). 19 cell lines were studied. Mean fold increase in aromatase activity (\pm SEM)

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was 2.69 ± 0.38 ($p < 0.02$ paired t test). In separate experiments on three cell lines the response to a range of concentrations of testosterone and DHT was studied. Testosterone gave a 2 fold increase in aromatase maximal at 10nmol. At the same concentration the response to DHT was over twice as great (4.3 fold). This response to testosterone and DHT was inhibited by cyproterone acetate (which blocks androgen receptors) indicating that androgen stimulation of skin aromatase is *via* an androgen receptor dependent mechanism.

- No aromatase was detected in hair follicle dermal papilla cells cultured from any of the above sites (14 cell lines). Testosterone failed to induce aromatase in dermal papilla cells.
- Dexamethasone stimulated aromatase 10-20 fold in fibroblasts (6 cell lines) and induced aromatase in dermal papilla cells (4 cell lines).
- Fibroblast aromatase was inhibited by 4OH-androstenedione, a known inhibitor of aromatase in other tissues. Kinetic studies in 3 cell lines were consistent with irreversible competitive inhibition. 50% inhibition occurred at approximately 25 nmol concentration.
- Aromatase mRNA was detected in skin fibroblasts (four cell lines) by the polymerase chain reaction (PCR). In spite of the absence of measurable enzyme activity we also detected aromatase mRNA in dermal papilla cells (four cell lines). Using a semi-quantitative

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dilutional technique the level of expression appeared greater in skin fibroblasts than in dermal papilla cells suggesting that the difference in enzyme activity between the two cell types is determined at the transcriptional level. PCR is an unreliable method for quantifying mRNA and we cannot exclude the possibility of post-transcriptional regulation. However, in experiments in which skin fibroblasts were incubated with conditioned media from dermal papilla cell cultures or were co-cultured with dermal papilla cells we found no evidence that dermal papilla cells contain or release factors which inhibit aromatase.

- To determine whether the results obtained using cell culture are representative of events occurring *in vivo* aromatase was measured by the tritiated water assay in skin samples from male and female scalp. Tissue was minced and then incubated with $1\beta^3\text{H}$ -androstenedione together with a source of NADPH. Each assay was done in the presence and absence of an aromatase inhibitor (4OH-androstenedione). Placental tissue was used as a positive control. Aromatase was detected in 9 of 12 samples from male frontal scalp by in only one of 5 samples from female scalp. The difference was statistically significant ($p < 0.01$).

(fmol/g	Mean	Median	SEM
Male scalp	91.7	44.5	32.8

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Female scalp	3.6	0	3.6
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Mean aromatase activity was greater in balding (110.0 fmol/g tissue/hour n=8) than in non-balding scalp (55.0fmol/g tissue/hour n=4) but the difference was not significant on this small number of samples. The addition of 25nmol 4OH-androstenedione reduced activity to background levels.

Our results support the original hypothesis. Aromatase activity is present in male scalp but is barely detectable or absent in female scalp. The cell culture studies show that skin aromatase is androgen dependent and this may be the explanation for the higher level of activity in males.

We believe we now have enough information to justify a pilot trial of a topical aromatase inhibitor in the treatment of male balding. We would use 4OH-androstenedione as its biochemistry and toxicity are well characterised and it is already licensed for use in humans (in the treatment of breast cancer).

Experimental

Cell Culture

Dermal papilla cells

Dermal papilla cell cultures were established using published methods (Messenger 1984, Messenger *et al* 1985). Briefly, human scalp and beard tissue was obtained as a by-product of the routine excision of benign skin lesions (e.g. naevi, cysts). Anagen hair follicles were removed by dissection. Dermal papillae were then isolated by microdissection under a stereomicroscope and transferred to the culture vessel (35mm plastic petri dish). Papillae were incubated in Dulbecco's minimal essential medium (DMEM) containing 10% pooled human serum at 37° in a humidified atmosphere containing 5%CO₂. Cell outgrowths occur after 3-14 days. Cells were cultured to confluence and then passaged into a 25cm² flask using standard cell culture techniques. Subsequent passaging was at a 3:1 split ratio.

Skin fibroblasts

Skin fibroblast cultures were established from the same skin samples as dermal papilla cultures. Interfollicular dermis was removed by dissection and cut into small pieces with a scalpel blade. The small tissue pieces were then placed in a 35mm plastic petri tissue culture dish and covered with culture medium (as above). Cell outgrowths occur after 3-14 and cultures were then handled as described above.

Messenger AG (1984) Cell culture of dermal papillae isolated from human hair follicles. *J Invest Dermatol* 82, 548

Messenger AG, Senior HJ, Temple A, Pope FM, Bleehen SS (1985) In vitro properties of dermal papilla cells cultured from human hair follicles. *Br J Dermatol* 113, 768

Aromatase Assay

A tritiated water release assay was used to measure aromatase activity in cells and tissue. The method has been published (Lephart & Simpson 1991). It relies on the fact that a hydrogen atom is released from the 1 β position during aromatisation of androstenedione.

- Cells were plated into a 24-well plate and cultured to confluence.
- The culture medium was decanted and the cells were washed with fresh serum-free medium.
- 1ml DMEM without serum containing the aromatase substrate was then added to each well.
- The substrate used was 1 β ³H-androstenedione. ³H released during aromatisation of 1 β ³H-androstenedione can be measured as tritiated water.

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- The cells were incubated with the substrate for 1-4 hours (usually 2 hours).
- The medium was decanted and extracted sequentially with chloroform and activated charcoal to remove unmetabolised substrate. Charcoal was removed by centrifugation and the radioactivity present (as $^3\text{H}_2\text{O}$) in aliquots of the aqueous supernatant were counted in a scintillation counter.
- The cell layer was removed with Triton X and the protein content measured using standard methods.
- To establish kinetic values for enzyme activity in each cell-line cells were incubated with the substrate in a range of concentrations (0.2 - 3.0 $\mu\text{Ci/ml}$). V_{max} and K_m were calculated from Lineweaver-Burke plots.
- In some experiments cells were incubated with testosterone and dihydrotestosterone in a range of concentrations for 24 hours prior to aromatase assay.
- In some experiments cells were incubated with dexamethasone in a range of concentrations for 18 hours prior to aromatase assay.
- In some experiments 4OH androstenedione (an inhibitor of aromatase) was added to the culture wells during the incubation with the aromatase substrate. A range of concentrations of the inhibitor and the substrate were used.

Lephart ED, Simpson ER (1991) Assay of aromatase activity. *Methods in Enzymology* 206, 477-483

Aromatase in tissue samples

The same basic technique was used to measure aromatase in tissue samples.

- Skin samples were obtained as a by-product of routine excision of benign lesions. Tissue was snap frozen in liquid nitrogen and stored in a liquid nitrogen freezer.
- Tissue was thawed, weighed and then minced with a scalpel blade.
- Tissue samples were incubated in serum free DMEM containing 1 $\mu\text{Ci/ml}$ $1\beta^3\text{H}$ -androstenedione and a source of NADPH. Incubations were for 4 hours.
- The medium was extracted and counted as described above.
- For each sample tissue was incubated with the substrate in the presence and absence of 25nmol 4OH androstenedione. Aromatase was calculated as the activity suppressible by 4OH androstenedione.

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Measurement of mRNA

- Cells were cultured to confluence in 75 cm² flasks.
- RNA was extracted using the method of Chomczynski and Sacchi (1987).
- cDNA was synthesised using Moloney murine leukaemia virus reverse transcriptase. The cDNA was used the polymerase chain reaction (PCR)
- PCR primers:
5'-TATCAGCAAGTCCTCAAGTATGTTC-3'
5'-TCAAGAAGAGCGTGTTAGAGGTGT-3'
- 10µl of the RT (reverse transcriptase) product was mixed with the following reagents:
10X reaction buffer 5µl
25mM MgCl₂ 3µl
Taq DNA polymerase (5u/µl) 0.4µl
Water 30.6µl
primers 15µM of each primer in water 0.5µl
- The mixtures were overlaid with mineral oil and subjected to 28 PCR cycles. The products were examined on 1.2% ethidium bromide stained agarose gel. PCR product was digested with Sac 1 to confirm its identity.
- For semi-quantification of mRNA, RT products were serially diluted twofold (i.e. 1,2,4,8,16,32).

Chomczynski P, Sacchi N (1987) Single step method of RNA isolation by acid guanidium-thiocyanate-phenol-chloroform extraction. *Analyt Biochem* 162 156-159

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Example 1

A typical formulation for a topical preparation containing an aromatase inhibitor is as follows:-

5% Propylene glycol

5 10% Ethanol or Isopropyl alcohol

85% Water

0.2-10% w/v 4-OH androstenedione

10 4-OH Androstenedione is dissolved in ethanol or isopropyl alcohol and propylene glycol. This solution is then diluted with water with stirring to provide a lotion suitable for application to the scalp.

Commercially available preservatives, such as one of the hydroxybenzoates, may optionally be added to increase shelf life of the product.

REFERENCES

1. Hamilton JB: Male hormone is a prerequisite and an incitant in common baldness, *Am J Anat* 71: 451, 1942.
2. Ebling FJG: The hormonal control of hair growth In: Orfanos CE, Happle R (eds.): Hair and Hair Diseases Berlin, Springer-Verlag, pp 267-299, 1990.
3. Jahoda CAB, Oliver RF: The dermal papilla and the growth of hair, in Orfanos CE, Happle R (eds): Hair and Hair Diseases, Berlin, Springer-Verlag, pp 19-44, 1990.
4. Messenger AG: Control of hair growth: an overview, *J Invest Dermatol* 101: 4s-9s, 1993.
5. Randall VA, Thornton MJ, Messenger AG: Cultured dermal papilla cells from androgen-dependent human hair follicles (e.g. beard) contain more androgen receptors than those from non-balding areas of scalp. *J Endocrinol* 133: 141-147, 1992.
6. Choudry R, Hodgins MB, Van der Kwast TH, Brinkmann AO, Boersma WJA: Localization of androgen receptors in human skin by immunohistochemistry: implications for the hormonal regulation of hair growth, sebaceous glands and sweat glands. *J Endocrinol* 133: 467, 1992.

-30-

7. Itami S, Kurata S, Sonoda T, Takayasu S: Mechanism of action of androgen in dermal papilla cells. Ann NY Acad Sci 642: 385-395, 1991.
8. Mohn MP: The effects of different hormonal states on the growth of hair in rats, in Montagna W, Ellis RA (eds): The Biology of Hair Growth, New York, Academic Press, pp 335-398, 1958.
9. Schweikert HU, Milewich L, Wilson JD: Aromatization of androstenedione by isolated human hairs. J Clin Endocrinol Metab 40: 413, 1975.
10. Sawawa ME, Penneys NS: Immunohistochemical distribution of aromatase and 3 β -hydroxysteroid dehydrogenase in human hair follicle and sebaceous gland. J Cutan Pathol 19: 309, 1991.
11. Stillman SC, Evans BAJ, Hughes IA: Androgen dependent stimulation of aromatase activity in genital skin fibroblasts from normals and patients with androgen insensitivity. Clin Endocrinol 35: 533-538, 1991.
12. Hutchinson JB: Aromatase: neuromodulator in the control of behaviour. J Steroid Biochem Molec Biol 44: 509, 1993.
13. Lephart ED, Simpson ER: Assay of aromatase activity. Methods in Enzymology 206: 477-483, 1991.

-31-

14. Simpson E, Lauber M. Demeter M et al: Regulation of expression of the genes encoding steroidogenic enzymes. J Steroid Biochem Molec Biol 40: 45-52, 1991.
15. Harada N: A unique aromatase (P-450arom) mRNA formed by alternative use of tissue-specific exons 1 in human skin fibroblasts. Biochem Biophys Res Comm 189: 1001-1007, 1992.

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CLAIMS

1. Use of an aromatase inhibitor as a cosmetic agent.
2. A method for treating or preventing hair loss comprising administering an aromatase inhibitor to an area to be treated.
- 5 3. A method according to Claim 2 wherein the aromatase inhibitor comprises any one or more of the inhibitors listed herein.
4. A method according to Claim 2 wherein the aromatase inhibitor comprises an antibody raised against at least a part of an aromatase enzyme.
- 10 5. An antibody for use in preventing hair loss, raised against an aromatase inhibitor.
6. Use of an aromatase inhibitor in the manufacture of a preparation for the reduction in the regression of hair growth or in the alleviation of baldness.
- 15 7. Use of an aromatase inhibitor as claimed in Claim 6 wherein the preparation is formulated for topical application with suitable excipients in the form of a lotion.
8. Use of an aromatase inhibitor as claimed in Claim 6 wherein the

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preparation takes the form of a cream, shampoo, conditioner or spray.

9. Use of an aromatase inhibitor as claimed in any of claims 6-8 wherein the aromatase inhibitor is selected because it is readily absorbed from the preparation into the skin and into the appropriate skin cells.
- 5 10. The use of an agent adapted to selectively bind to an androgenic receptor, such that the receptor mediated androgenic control of aromatase is suppressed or prevented, as a cosmetic agent.
- 10 11. Use of an agent as claimed in Claim 10 wherein said agent is an antagonist able to bind to said receptor but unable to produce a receptor mediated response.
- 15 12. Use of an agent according to Claim 11 wherein the said antagonistic agent may be synthetically produced or naturally occurring or alternatively a manipulated form of the naturally occurring androgen, for example, it may comprise a form of the naturally occurring androgen which is able to bind, and so block, the relevant receptor but unable to produce a receptor mediated response.
13. A method for treating or preventing hair loss comprising administering an androgen receptor antagonist to an area to be treated.
- 20 14. A medicament and/or cosmetic agent comprising an aromatase inhibitor and an androgen receptor antagonist.

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15. A medicament and/or cosmetic agent as claimed in Claim 11 wherein the aromatase inhibitor and the androgen receptor antagonist will be provided in a ratio that is related to the cellular concentrations of the corresponding aromatase and receptor.
- 5 16. A medicament and/or cosmetic agent as claimed in Claim 14 or Claim 15 wherein said inhibitor is an anti-body, polyclonal or monoclonal, which has been raised against at least a part of the aromatase enzyme.
- 10 17. Use of an androgen receptor antagonist in the manufacture of a preparation for the reduction in the regression of hair growth or in the alleviation of baldness.
18. Use of a preparation as claimed in Claim 17 wherein the preparation is formulated for topical application with suitable excipients in the form of a lotion.
- 15 19. Use of a preparation as claimed in Claim 17 wherein the preparation may take the form of a cream, shampoo, conditioner or spray.
- 20 20. Use of a preparation as claimed in any of claims 17-19 wherein the antagonist is selected because it is readily absorbed from the preparation into the skin and into the appropriate skin cells.
21. A method for treating or preventing hair loss comprising

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administering either successively or simultaneously an aromatase inhibitor and an androgenic receptor antagonist.

- 5 22. A method for treating or preventing hair loss as claimed in Claim 21 wherein said aromatase inhibitor is any one or more of the inhibitors listed herein such as, for example, 4-OH androstenedione.
23. A method for treating or preventing hair loss as claimed in Claim 21 wherein the androgenic receptor antagonist is, for example, cyproterone acetate.
- 10 24. A method of detecting if an individual is predisposed to hair growth regression or baldness by taking a sample of cells from various skin sites prone to baldness on the individual, culturing these cells, measuring their aromatase activity and comparing this activity with control values for people within a control group and values from people who have suffered from hair loss.
- 15 25. Use of the method as claimed in Claim 24 for detecting whether an individual is likely to benefit from treatment with an aromatase inhibitor preparation as claimed herein.
26. Use of a pre-oestrogen in the manufacture of a preparation for the treatment of hirsutism.

INTERNATIONAL SEARCH REPORT

 International Application No
 PCT/GL 95/02166

 A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 A61K7/06 C07K16/40

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,85 02543 (KASZYNSKY, EDWIN, G.) 20 June 1985 *Abstract*	10-21,23
A	WO,A,86 02269 (HANDELMAN, JOSEPH) 24 April 1986 *Abstract*	10-21,23
A	EP,A,0 566 979 (ZINGRAF, INGEBORG) 27 October 1993 *Abstract*	10-21,23
A	EP,A,0 163 490 (MORTIMER, CHRISTOPHER HARRY, DR.) 4 December 1985 *abstract*	10-21,23
	--- -/--	

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Date of the actual completion of the international search

8 January . 1996

Date of mailing of the international search report

15. 01. 96

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/GI 15/02166

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,86 01402 (SCHERING AKTIENGESELLSCHAFT) 13 March 1986 *Abstract* ---	10-21,23
A	DE,A,28 40 144 (GÖDECKE AG) 27 March 1980 *Ansprüche* ---	10-21,23
A	US,A,4 684 635 (NORMAN ORENTREICH ET AL) 4 August 1987 *Abstract* ---	10-21,23
A	PATENT ABSTRACTS OF JAPAN vol. 10, no. 166 (C-353) (2222) 13 June 1986 & JP,A,61 018 711 (POLA KASEI KOGYO K.K.) 27 January 1986 see abstract ---	10-21,23
A	PATENT ABSTRACTS OF JAPAN vol. 11, no. 317 (C-452) (2764) 15 October 1987 & JP,A,62 103 005 (KANEBO LTD) 13 May 1987 see abstract ---	10-21,23
A	DE,A,36 21 757 (WELLA AG) 14 January 1988 *Abstract* ---	10-21,23
A	DE,A,36 15 396 (WELLA AG) 12 November 1987 *Abstract* ---	10-21,23
A	JOURNAL OF STEROID BIOCHEMISTRY, vol.83, no.19, 1982 pages 587 - 590 F. J. G. EBLING ET AL *Article* ---	21,22
A	J. CLIN. ENDOCRINOL. METAB., vol.74, no.39(6, 1972 pages 1012 - 1019 HANS U. SCHWEIKERT *Article* ---	21,22
A	J. CLIN. ENDOCRINOL. METAB., vol.74, no.38(5, 1972 pages 811 - 819 HANS U. SCHWEIKERT ET AL *Article* ---	21,22
A	J. ENDOCRINOL., vol.73, no.59(2, 1972 pages 345 - 351 J.S. JENKINS ET AL *Article* -----	21,22

INTERNATIONAL SEARCH REPORT

International Application No

PCT/G. 35/02166

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-8502543	20-06-85	AU-B- 584070	18-05-89
		AU-B- 3745885	26-06-85
		CA-A- 1251737	28-03-89
		DE-A- 3486090	08-04-93
		DK-B- 170726	27-12-95
		EP-A, B 0165970	02-01-86
		JP-B- 7045382	17-05-95
		JP-T- 61500966	15-05-86
		US-A- 4885289	05-12-89
WO-A-8602269	24-04-86	US-A- 4720489	19-01-88
		AU-B- 590730	16-11-89
		AU-B- 4867385	02-05-86
		CA-A- 1262335	17-10-89
		DE-A- 3585526	09-04-92
		EP-A, B 0198893	29-10-86
		JP-B- 6053680	20-07-94
		JP-T- 62500932	16-04-87
		NO-B- 174832	11-04-94
EP-A-0566979	27-10-93	DE-A- 4213314	28-10-93
EP-A-0163490	04-12-85	AU-B- 4405185	13-12-85
		CA-A- 1257838	25-07-89
		WO-A- 8505270	05-12-85
		JP-T- 61502188	02-10-86
WO-A-8601402	13-03-86	DE-A- 3432584	24-04-86
		DE-A- 3442857	22-05-86
		AU-B- 4805085	24-03-86
DE-A-2840144	27-03-80	NONE	
US-A-4684635	04-08-87	CA-A- 1243609	25-10-88
		US-A- 5053403	01-10-91
DE-A-3621757	14-01-88	NONE	
DE-A-3615396	12-11-87	NONE	